Isolation of the *hreX* Gene Encoding the HR Elicitor Harpin (Xcp) from *Xanthamonas campestris* pv. *pelargonii*. S. SWANSON and Z-M. Wei. EDEN Bioscience Corporation, Bothell, WA 98011 USA. Phytopathology 90:S75. Publication no. P-2000-0537-AMA.

This study reports the isolation of a gene encoding a proteinaceous HR elicitor from *Xanthomonas campestris* pv. *pelargonii, Xcp*. The HR elicitor exhibits a high potency for eliciting HR in tobacco. Treatment of the *Xcp* HR Elicitor with proteases resulted in a loss of HR activity. Degenerate oligonucleotides were designed based on amino acid sequences obtained from the purified HR elicitor and used to screen a *Xanthomonas campestris* pv. *pelargonii* genomic library. An open reading frame, ORF, was identified consisting of 381 base pairs that encoded a protein of 126 amino acids. The ORF initiated with a typical methionine start codon and was preceded by a putative ribosome-binding site. The ORF was designated as the *hreX* gene, encoding the HR elicitor harpin (Xcp). HreX has a molecular weight of 13.3KD, a theoretical pI of 3.8 and is glycine rich. Further studies of harpin (Xcp) and its bioactivity are currently underway.

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Introduction

The last decade has been witness to the identification, isolation and characterization of a number of hypersensitive response elicitors from plant pathogens (HR elicitors). HR elicitors have been reported from species of *Erwinia*, *Pseudomonas*, and *Ralstonia*. For the most part, the HR elicitors reported have been classified as belonging to the harpin protein family. Harpin proteins elicit the hypersensitive response in tobacco and other non-host plants, and are glycine rich, and heat-stable. They are secreted via a type III secretion system. Harpin proteins are generally located in or approximate to an *hrp* gene cluster, a highly conserved region of genes that encode regulatory factors and other components of the type III secretion system. In most of the species above, identification of the *hrp* clusters preceded the identification of the harpin proteins. The *hrp* gene clusters from several species of *Xanthomonas* have also been identified and highly studied, though to date no HR elicitor from *Xanthomonas* has not been reported. The current study reports the isolation of a gene encoding an HR elicitor from *Xanthomonas campestris pv. pelargonii* (*Xcp*).

Proteinaceous HR Elicitor

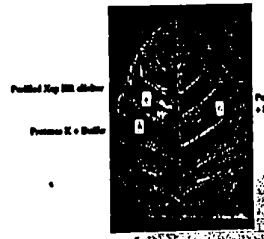

Figure 1.
Wild type *Xcp* was grown in a minimal media that was optimized to induce the expression of an HR elicitor. The induced culture was centrifuged, and the cell pellet resuspended and sonicated. The sonicate was then heat-treated, cooled and centrifuged. The resulting supernatant, or cell free elicitor preparation (CFEP), was infiltrated into tobacco leaves and a hypersensitive response was observed. When samples of the CFEP were incubated in the presence of a protease prior to infiltration, no development of HR symptoms was observed in the tobacco plants.

Identification and Isolation of HR Elicitor

Figure 2.
CFEP was used as the starting material for the chromatographic purification of the HR elicitor. Purification consisted of two hydrophobic interaction steps: a cation exchange step, and a reverse phase chromatography polishing step (RPC). The final RPC step yielded a nearly homogeneous *Xcp* HR elicitor.
Lane A, CFEP loaded on to Butyl Seph.; lane B, HR active fraction from the Butyl Seph. loaded onto Mono S; lane C, HR active fraction from Mono S loaded on to Phenyl Seph.; lane D, HR active fraction from Phenyl Seph. loaded on to an RPC column; lane E, HR active fraction from the final RPC step, representing the purified *Xcp* HR elicitor.

Gene Isolation

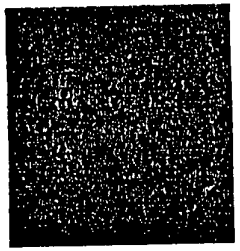

Figure 3.
The purified HR elicitor was proteolytically digested and the resulting peptides separated. Amino acid sequencing of one of the peptides yielded an unambiguous sequence of nine amino acids. A fully degenerate pool of oligonucleotides was made based on the amino acid sequence. The oligonucleotide pool was 5' end radiolabeled and used as a probe against an *Xcp* genomic library.
The *Xcp* genomic library was constructed in pBluescript II that contained 9 to 12 kb *Xcp* Sau3 A1 genomic DNA fragments. The library was plated and the DNA transferred to nylon membranes and fixed. The membranes were hybridized with the previously described probe. Approximately 7000 colonies were screened, and 30 colonies were isolated for further analysis. Examples of positive hybridizing DNA from library constructs are shown.

Figure 4.
Plasmids were isolated from colonies that showed positive hybridization with the HR elicitor probe. A Southern analysis was performed on the digested plasmids in order to confirm the purity of the colony hybridization (Figure 5). Samples 2, 4, and 6 were further analyzed.

Figure 5.
Genomic DNA inserts from samples 2, 4, and 6 (Figure 4) were digested and subcloned. The subclones were constructed in pBluescript II and contained 1.5 to 3 kb genomic inserts. A final Southern analysis was performed on the subclones. This confirmed the presence of the genomic DNA fragments which hybridized with the HR elicitor probe.

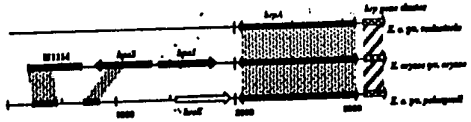

Figure 6.
DNA sequencing was performed on the inserts of the final constructs which hybridized with the HR elicitor probe (Figure 5; samples 2a, 4a, and 6a). A 378 base pair open-reading-frame (ORF) encoded a 126 amino acid protein was identified. The deduced protein had a molecular weight of ca. 12 kDa, a theoretical pI of 3.5, and was glycine rich. These characteristics match those of the originally purified *Xcp* HR elicitor protein. The ORF was designated *hreX*. The *hreX* gene product, HreX, consisted of 15.8% glycine, 18.4% glutamine, 11.4% serine, and one cysteine residue (0.9%). Sequencing of the regions flanking of *hreX* revealed that *hreX* was located immediately downstream of a *hrpA* homologue. Based on *hrp* gene clusters reported for other *Xanthomonas* species, it would seem likely that *hreX* is located just outside of the *hrp* gene cluster. BLAST analysis did not identify any genes with significant sequence similarities to the *hreX* gene. Significant homologies were observed between regions upstream of the *hreX* gene and BH114 and *hpa2* from *X. oryzae pv. oryzae* (1).

hreX Homologue

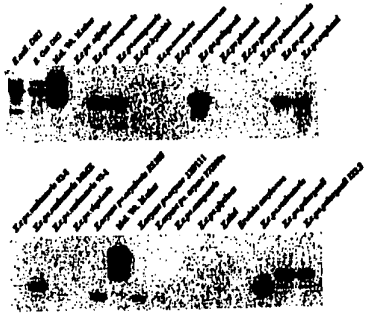

Figure 7.
Various species of *Xanthomonas* and other bacteria were obtained and screened for the presence of an *hreX* homologue. Southern analyses were performed with *hreX* probe. Hybridization with the *hreX* probe was observed in the genomic DNA of the bacterial species appearing in bold type.

Bioactivity of HreX

HreX was expressed in *E. coli*, and prepared in an identical fashion to the previously described CFEP, to generate a crude HreX extract. When infiltrated into tobacco and several other plant species, the HreX extract induced the hypersensitive response. Experiments have also been conducted with crude HreX extract to evaluate its ability to induce resistance to tobacco mosaic virus and to induce plant growth enhancement in tomato. Three days after the treatment of tobacco plants with the crude HreX extract, tobacco plants were inoculated with tobacco mosaic virus (TMV). Four days after TMV inoculation, the treated plants were observed to have 75% fewer TMV-caused lesions compared to the buffer treated control plants. Tomato plants grown from seed soaked in the crude HreX extract were observed to have an average growth 15-20% greater than the buffer treated control plants (determined by measuring plant height).

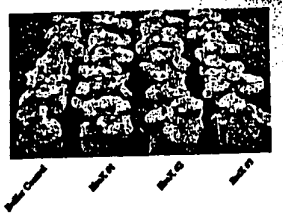

Figure 8.
Additional experiments were conducted with HreX to evaluate its ability to induce disease resistance in geraniums. Geraniums (variety Rose Star) were treated with varying concentrations of a crude HreX extract (HreX #1, 2 & 3). Five days after treatment, the geranium plants were inoculated with *Xanthomonas campestris pv. pelargonii* (100 ml at 1×10^6 cfu per ml). Fifteen days after inoculation, the HreX solution was determined to be effective in controlling the disease.

Conclusions

- A hypersensitive response elicitor was purified to near homogeneity from *Xanthomonas campestris pv. pelargonii*.
- The gene corresponding to the *Xcp* HR elicitor was isolated and designated *hreX*.
- The *hreX* gene is located next to *hrpA* just outside of the hrp gene cluster.
- BLAST analysis did not identify any genes with significant sequence similarities to the *hreX* gene. Significant homologies were observed between regions upstream of the *hreX* gene and BH114 and *hpa2* from *X. oryzae pv. oryzae* (1).
- Sequences similar to *hreX* have been identified by Southern analysis in various other species of *Xanthomonas* including some pathovars of *X. c. pv. vesicatoria* and *X. oryzae*.
- HreX was expressed in *E.coli* and induced a hypersensitive response when infiltrated into tobacco.
- Treatment of tobacco and geranium plants with a crude HreX extract resulted in increased disease resistance.
- Treatment of tomato plant with the HreX extract resulted in plant growth enhancement.
- Studies are currently underway to determine whether HreX is secreted via the type III secretion system.

1. Zhu, W., Magbanua, M. M., and P. F. White. 2000. Identification of Two Novel *hrp*-Associated Genes in *hrp* Gene Cluster of *Xanthomonas oryzae pv. oryzae*. J. Bacteriol. 179:5655-5662.